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Safety and Immunogenicity of Live Oral Cholera Vaccine Candidate CVD 110, a Δ*ctxA* Δ*zot* Δ*ace* Derivative of El Tor Ogawa *Vibrio cholerae*

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The current pandemic of cholera is caused primarily by *Vibrio cholerae* O1 of the El Tor biotype. Live attenuated classical biotype *V. cholerae* vaccine strains prevent severe and moderate cholera due to either biotype in challenged volunteers but may provide less protection against mild cholera due to El Tor organisms. CVD 110, a new *ctxA*-deleted vaccine strain derived from an El Tor Ogawa parent, lacks zona occludens toxin (Zot), accessory cholera enterotoxin (Ace), and hemolysin/enterotoxin. Ten healthy adult volunteers were given 10^8 cfu of CVD 110 with buffer; 7 developed diarrhea (mean stool volume, 861 mL). Vaccine organisms were shed in stool by all vaccinees and were recovered from duodenal fluid in three-quarters of vaccinees. After vaccination, the geometric mean peak reciprocal vibriocidal titer among vaccinees was 17,829. CVD 110 is a powerful immunogen but insufficiently attenuated despite the absence of known potential enterotoxins of *V. cholerae*. Another unrecognized toxin or colonization alone may be responsible for diarrhea after ingestion of this strain.

The severe purging of cholera characterized by the passage of voluminous rice water stools (~1 L/h in adults) is due to the effects of cholera enterotoxin (CT). This toxin consists of one enzymatically active A subunit (encoded by *ctxA*) and five nontoxic but immunogenic B subunits (encoded by *ctxB*). Attenuated *Vibrio cholerae* O1 strains can be prepared by precise deletion of genes that encode CT, the primary virulence property, leaving intact the various other antigens involved in stimulating a protective immune response.

Several such vaccine strains have been created from pathogenic parent strains whose ability to cause cholera and to stimulate protective immunity has been documented in volunteer studies [1]. JBK 70 is a Δ*ctxA* Δ*ctxB* derivative of virulent El Tor Inaba strain N16961 in which the genes encoding both the A and B subunits of CT were deleted (Δ) by recombinant techniques. While notably attenuated compared to its wild-type parent, JBK 70 nevertheless caused mild diarrhea in 6 of 14 recipients of the vaccine, accompanied by malaise, abdominal cramps, nausea, and headache. A Δ*ctxA* derivative of virulent classical Ogawa strain 395, CVD 101, was fed to volunteers to determine whether genetically engineered vaccine strains derived from classical parent strains might be better tolerated than El Tor derivatives. Un-

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Written informed consent was obtained from the volunteers enrolled in the studies, and the human experimentation guidelines of the US Department of Health and Human Services and of the University of Maryland at Baltimore's Institutional Review Board were followed in the conduct of the clinical research.

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like JBK 70, this strain expressed the immunogenic B subunit of CT. At every dosage, CVD 101 colonized well and elicited excellent vibriocidal and antitoxin responses, but again, a proportion of the vaccinees had mild diarrhea, malaise, and abdominal cramps [1].

In the search for a less reactogenic vaccine strain, the *ctxA* gene was next deleted from wild-type classical Inaba *V. cholerae* 569B, also known to cause cholera in volunteers [2]. Notably, strain 569B was the only strain examined that did not produce a cytotoxin that could be neutralized by antiserum prepared against Shiga toxin [3]. The resultant vaccine strain, CVD 103, was well tolerated in volunteers, and the vibriocidal and antitoxin responses were prominent [2]. CVD 103 conferred significant protection against challenge with *V. cholerae* of either classical or El Tor biotype and either Inaba or Ogawa serotype [2]. A further derivative, CVD 103-HgR, was constructed with a gene encoding resistance to Hg^{++} ions inserted into the El Tor hemolysin gene locus. The Hg^{++} resistance provides a helpful marker that can be used to differentiate the vaccine strain from wild-type *V. cholerae* classical Inaba strains. CVD 103-HgR was well tolerated and protected volunteers against experimental challenge with El Tor Inaba N16961, El Tor Ogawa E7946 (~60% protective efficacy against any diarrhea caused by either strain), and classical Inaba 569B (100% protective efficacy) [2, 4, 5].

Although CVD 103 and CVD 103-HgR provide 100% protection against severe cholera of both classical and El Tor biotypes, a minority of recipients of these classical biotype vaccine strains develop mild diarrhea when challenged with El Tor organisms (~60% protective). In the current pandemic, *V. cholerae* of the El Tor biotype are predominant, and there are biotype-specific antigens that may contribute to protective efficacy. Therefore, an El Tor strain that does not cause diarrhea and provides protection against the most epidemiologically important biotype of *V. cholerae* may be desirable.

CVD 110 is a newly constructed *ctxA*-deleted, Hg^{++}-resistant strain derived from El Tor Ogawa E7946. Unlike the reactogenic El Tor *V. cholerae* vaccine strain JBK 70, CVD 110 lacks the newly described zona occludens toxin (Zot) and accessory CT (Ace), which may account for the diarrheagenicity of JBK 70 and CVD 101. Zot is a toxin present in the supernatant of *V. cholerae* cultures that affects intercellular tight junctions (zona occludens) of intestinal epithelial cells, rendering them permeable to solute [6]. The activity of Zot is measured in Ussing chambers in which the potential difference, short-circuit current, and tissue conductance across rabbit ileum are determined. Zot causes an increase in tissue conductance, which is a sensitive measure of the functional integrity of the intercellular tight junctions [6]. Zot allows solute to permeate the paracellular space [6]. Ace is a distinct toxin that causes an increase in potential difference in Ussing chambers and secretion in rabbit ligated ileal loops [7]. The protein sequence of Ace is similar to eukaryotic ion transporting ATPases. CT, Zot, and Ace comprise a genetic virulence cassette that may contribute to the full pathogenic potential of *V. cholerae*.

We sought to determine the clinical and immunologic response to an El Tor strain lacking the four putative *V. cholerae* enterotoxins (El Tor hemolysin, CT, Zot, and Ace).

Materials and Methods

Volunteers. Healthy adults, 18 to 40 years old, from the Baltimore community were recruited to participate. Volunteers were screened to confirm their good health by medical histories and physical examinations, a battery of clinical hematology and chemistry tests, electrocardiograms, and serologic tests for syphilis, hepatitis B surface antigen, and human immunodeficiency virus. Before vaccination, stools were examined for bacterial pathogens, ova, and parasites.

Vaccine. *V. cholerae* vaccine strain CVD 110 was grown, lyophilized, and packaged at the Swiss Serum and Vaccine Institute, Berne, Switzerland. The formulation consisted of sachets containing a single dose ($3–5 \times 10^8$ viable organisms) of lyophilized vaccine and 25 mg of aspartame. An accompanying sachet contained an effervescent buffer powder consisting of 2.5 g of sodium bicarbonate and 1.65 g of ascorbic acid. The vaccine was stored at 4°C before use.

Vaccination. Volunteers were admitted to the Research Isolation Ward of the Center for Vaccine Development located in the University of Maryland Hospital. After a 48-h period of acclimatization, 16 volunteers were randomized to receive a single oral dose of $\sim 3–5 \times 10^8$ cfu of CVD 110 ($n = 10$) or an identical appearing placebo ($n = 6$) consisting of buffer alone. Placebo recipients were included to allow comparison of adverse effects and to monitor person-to-person transmission of vaccine organisms among adults living in the isolation ward.

At the time of vaccination, a sachet of buffer was added to 100 mL of distilled water in a cup. A sachet containing a dose of vaccine was then added to the buffer to make a vaccine cocktail. The volunteers then ingested the cocktail. Volunteers had nothing to eat or drink for 90 min before and after vaccination.

Volunteers were closely monitored in the isolation ward for 12 days after vaccination; vital signs were measured every 6 h. All stools from each volunteer were collected in plastic containers, examined, and graded on a five-point scale [1], and the volume was measured when the stool was loose. Volunteers were interviewed daily by a physician and asked about symptoms. Diarrhea was defined as two or more loose stools within 48 h totaling ≥200 mL in volume or a single loose stool ≥300 mL in volume. Antibiotic therapy (500 mg tetracycline every 6 h for 5 days) was given before discharge.

Bacteriology. Every stool passed by volunteers after vaccination was cultured daily for the vaccine strain. Stool was plated directly onto thiosulfate citrate bile salts sucrose (TCBS) agar and into alkaline peptone water enrichment broth for overnight incubation before plating onto TCBS agar. Up to three stools

each day were cultured quantitatively to determine the number of vaccine organisms per gram of stool.

At ~20 and 44 h after vaccination, fasting volunteers swallowed gelatin capsules containing string devices (Entero-Test; HDC, Mountain View, CA) to collect samples of bile-stained duodenal fluid. After 4 h, the strings were removed and the color and pH of the distal 15 cm were determined. Duodenal fluid was squeezed from the end of the string and cultured as described above.

Immunology. Serum samples were obtained before vaccination and on days 7, 10, 21, and 28 after vaccination. Titers of vibriocidal antibodies were measured in serum [4] as well as antibody responses to *V. cholerae* lipopolysaccharide (LPS) and CT by ELISA [4]. A fourfold rise in titer was considered significant.

Before and on days 7 and 10 after vaccination, heparinized blood was collected for antibody secreting cell (ASC) determinations. ASC of the IgG, IgM, and IgA classes against *V. cholerae* O1-specific Inaba LPS and CT were measured by ELISPOT as previously described [8]. Peripheral blood mononuclear cells were separated by ficoll-hypaque density gradient centrifugation (Organon Teknika, Durham, NC) and added to antigen-coated or blank microtiter plates at a concentration of 0.25×10^6 cells/well. Wells were run in quadruplicate for each antigen-immunoglobulin class. Data were expressed as the number of specific ASC per 10^6 mononuclear cells. Four or more spots on the reaction plate were considered significant.

Table 1. Immunologic responses to *V. cholerae* vaccine strain CVD 110.

Assay	No. of responders (n = 10)	Response (range)
Ogawa vibriocidal antibody (GM peak reciprocal titer)*	10	17,829 (10,240–20,480)
Inaba vibriocidal antibody (GM peak reciprocal titer)*	10	3,152 (80–20,480)
Anti-CT (GM peak net OD)*	9	1.58 (0.59–2.82)
Antibody secreting cells (GM peak no. of spots/10^6 PBMC)*†		
IgA anti-Ogawa LPS	9	161 (36–316)
IgM anti-Ogawa LPS	9	101 (20–402)
IgA anti-Inaba LPS	9	31 (4–270)
IgM anti-Inaba LPS	6	60 (4–416)
IgA anti-CT	8	38 (4–360)
IgM anti-CT	3	14 (6–36)

NOTE. CT, cholera toxin; GM, geometric mean; LPS, lipopolysaccharide; OD, optical density; PBMC, peripheral blood mononuclear cells.

* Measured at 7 and 10 days after vaccination.

† On day 0, ≤3 cells producing antibody against any of the 3 antigens were detected.

Results

Clinical responses to vaccination. Eight of 10 vaccinees had adverse reactions beginning 1–5 days after ingestion of CVD 110. Seven of 10 vaccinees and none of 6 placebo recipients developed diarrhea (thick liquid and opaque watery stools). The mean number of stools was 6 (range, 2–13). The three volunteers who did not have diarrhea did not have preexisting ASC against *V. cholerae* Ogawa or Inaba LPS or preexisting vibriocidal antibody. The mean diarrheal stool volume was 861 mL (range, 377–1687). An additional vaccinee had fever (maximum temperature, 38.5°C), anorexia, malaise, headache, and abdominal cramps on days 1 and 2 after vaccination, without diarrhea.

Recovery of vaccine organisms in duodenal fluid and stool. Vaccine organisms were recovered in the stool cultures of all vaccinees for a mean of 7 days beginning in the first 72 h after vaccination. The geometric mean peak excretion was 2.0×10^7 cfu/g. CVD 110 was recovered from duodenal string fluids in 6 of 8 vaccinees who had at least 1 adequate specimen. No vaccine organisms were recovered from the stools or duodenal fluids of placebo recipients.

Serologic responses to vaccination. After vaccination with a single oral dose of 10^8 cfu of CVD 110, all 10 vaccinees developed fourfold or greater rises in serum Ogawa (homologous serotype) and Inaba (heterologous serotype) vibriocidal antibodies with a geometric mean peak reciprocal titer after vaccination of 17,829 and 3152, respectively (table 1). After vaccination, 9 of 10 vaccinees who received CVD 110 developed significant rises in serum IgG antibody against CT (table 1).

ASC assays. ASC responses to Ogawa and Inaba LPS and to CT were sought. All 10 vaccinees developed ASC to Ogawa and Inaba LPS, or both (table 1). Nine of 10 vaccinees developed IgA anti-Ogawa LPS ASC with a geometric mean peak number of 161 cells/10^6 peripheral blood mononuclear cells among responders (table 1). Eight of 10 vaccinees developed IgA anti-CT B subunit with a geometric mean peak number of 38 cells among responders. One placebo recipient had 6 ELISPOTS, indicating IgA anti-Inaba LPS on day 10 after vaccination; no other ASC responses occurred among placebo recipients.

Discussion

Three known potential enterotoxins (CT, Zot, Ace) are encoded on a virulence cassette in the *V. cholerae* chromosome. El Tor strains also elaborate a hemolysin that has enterotoxic activity [9]. Deletion or inactivation of these four virulence factors in strain E7946, however, yielded a strain that still caused mild-to-moderate diarrhea in adult volunteers. Moreover, deletion of a putative colonization factor (core-encoded pilin) encoded on this cassette [10] appar-

ently did not reduce colonization to a clinically meaningful extent. In previous studies in which volunteers received a 300- to 500-fold smaller dose (10^6 cfu) of *V. cholerae* E7946, the fully virulent parent strain of CVD 110, the diarrhea attack rate was 88%–100%, with a mean diarrheal stool volume of 4.7 L ([11], unpublished data). Therefore, deletion of CT, Zot, and Ace may have attenuated the parent wild-type strain, but only partly.

In initial clinical studies of *V. cholerae* mutated in the *ctx* gene, we proposed two possible explanations for the ability of these strains to cause diarrhea and other symptoms [1]. The first hypothesis was that diarrhea after ingestion of CT-deleted strains was due to an enterotoxin distinct from CT that had not yet been recognized. The second hypothesis was that diarrhea was due solely to colonization of the small bowel by attenuated *V. cholerae*. It is not clear which explanation accounts for the diarrheagenicity of CVD 110.

The assumption that all potential enterotoxins had been removed from *V. cholerae* CVD 110 was based on Ussing chamber studies showing that culture supernatants of CVD 110 caused no change in short circuit current (Isc) in rabbit ileal tissue. Genes for three toxins, CT, Zot and Ace, which had been shown to increase Isc, had been removed from CVD 110. In addition, the gene for the hemolysin/cytotoxin of *V. cholerae*, which has been shown to cause fluid accumulation in ligated rabbit ileal loops [12], was also mutated in CVD 110. The results of the volunteer study suggest that there is yet another toxin(s) produced by *V. cholerae* that can cause diarrhea and other symptoms. It was suggested previously that a Shiga-like toxin may be responsible for the reactogenicity of CT-deleted strains of *V. cholerae*, but this has never been proven [3].

The Ussing chamber is a classic technique for detecting and characterizing enterotoxins, and there is significant literature on the activity of CT and *Escherichia coli* heat-stabile enterotoxin in increasing Isc in Ussing chambers. If there is another enterotoxin produced by CVD 110, there are a number of potential reasons why supernatants from CVD 110 cultures did not alter Ussing chamber activity: The toxin may not be active on rabbit tissue but may be active on human tissue; the culture conditions used to grow CVD 110 for Ussing chamber assays may not favor expression of the toxin; the toxin may not be present in the supernatant as are CT, Zot, and Ace but instead may be cell-associated and unlikely to be detected in culture supernatants; or the toxin may be electrogenically silent (such as the Shiga toxin produced by *Shigella dysenteriae*) [13].

An alternative explanation for the residual diarrheagenic potential of CVD 110 is that avid adherence to enterocytes of the proximal small bowel may somehow result in secretion leading to diarrhea. This hypothesis is supported by experiments of Smith and Linggood [14], who demonstrated that in piglets, *E. coli* strains that express both a colonization factor (K88) and enterotoxin cause diarrhea. Moreover, *E. coli* strains that had lost the enterotoxin but still expressed K88 pili were able to cause diarrhea in about one-third of infected animals. Using a strain of enterotoxigenic *E. coli* originally isolated from a patient, Wanke and Guerrant [15] showed that a derivative that had lost the enterotoxin but still possessed colonization factor antigen/II could cause diarrhea in the rabbit reversible ileal tie model. These authors suggest that normal metabolic or enzymatic products of the colonizing bacteria might act directly in the gut as secretagogues or as agents capable of altering gut absorption or gut motility acting through the enteric nervous system. A subsequent study by these investigators showed that colonization of the ileum with this nontoxigenic *E. coli* strain caused reversal of the normal net ileal absorption to net secretion of water, sodium, and chloride as well as a significant decrease in mucosal sucrase activity [16]. We have previously shown that the same strain used by these investigators also caused mild diarrhea in 2 of 19 volunteers who ingested 10^9 or 10^{10} cfu of organism [17]. However, it is difficult, if not impossible, to determine with certainty that these putatively nontoxigenic strains do not produce a toxin that is undetectable by the conventional assays and growth conditions used.

While it is difficult to accurately assess intestinal colonization in volunteer studies, the extremely high vibriocidal antibody levels and long duration of stool shedding seen with CVD 110 indicate that this strain colonizes the human intestine very well. "Colonization" may include two types of interaction of vaccine organisms with mucosal cells: antigenic uptake by M cells overlying the gut-associated lymphoid tissue (which leads to an immune response) and adherence to enterocytes (which may lead to diarrhea). The vigorous immune response after ingestion of CVD 110 indicates that CVD 110 attaches to and is taken up by M cells; it is not clear whether this process or adherence to enterocytes resulted in diarrhea. In our small study, volunteers with negative duodenal fluid cultures nevertheless had large volumes of diarrhea and excellent immune responses, suggesting that both types of colonization occurred. CVD 103-HgR does not colonize the human intestine as well as does CVD 110 but is highly immunogenic; perhaps the lack of reactogenicity of CVD 103-HgR is in part due to decreased adherence to enterocytes. Future studies in search of additional *V. cholerae* toxins are ongoing, and the search for a nonreactogenic El Tor vaccine strain will continue.

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